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Resveratrol Modulates and Reverses the Age-Related Effect on Adenosine-Mediated Signalling in SAMP8 Mice

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Abstract

Resveratrol (RSV) is a natural compound present in berries, grapes and red wine that has shown some neuroprotective properties, but the mechanism by which RSV exhibits its protective role is not very well understood yet. Little is known about the effect of RSV on adenosinergic system, a system regulated in an age-dependent manner in SAMP8 mice, widely considered as an Alzheimer's model. Therefore, the aim of the present work was to assess whether RSV intake was able to modulate the adenosine-mediated signalling in SAMP8 mice. Data showed herein clearly demonstrate the ability of RSV to modulate adenosine receptor gene expression as well as transduction pathway mediated by receptors expressed on plasma membrane. Interestingly, this polyphenol was able to reverse the age-related loss of adenosine A_1 receptors and its corresponding signalling pathway. Moreover, adenosine A_{2A} receptors were not modulated by aging or RSV, but A_{2A}-mediated signalling was completely desensitized after RSV treatment compared to untreated mice. Enzymes involved on adenosine metabolism, such as 5′-nucleotidase and adenosine deaminase, were found to be reduced after RSV treatment, but adenosine levels remained unchanged. Nevertheless, an age-related decrease on 5′-nucleotidase activity and adenosine and related metabolite levels was observed. In conclusion, our data show that RSV modulates adenosine-mediated signalling, strongly suggesting that the role of RSV via adenosine receptor signalling and its modulation of neurotransmission in neurodegenerative diseases should be considered as new therapeutic target for RSV neuroprotective effect.

Keywords Resveratrol · Adenosine signalling · Aging · Alzheimer's disease · SAMP8 mice

Introduction

Neurodegeneration is one of the main challenges for the next future due to the expected over cost caused by the longer lifespan of the population in which the major risk factor for neurodegenerative diseases is the age. Adenosine is a purine nucleoside widely distributed in the body, whose effects are mediated through adenosine receptors (ARs). These receptors belong to the G protein-coupled receptor (GPCR) family, and they have been classified into A_1, A_{2A}, A_{2B} and A_3 [1]. Adenosine is fine tune regulated by 5′-nucleotidase and adenosine deaminase, which are involved on adenosine production and degradation, respectively [2]. This nucleoside, together with their receptors, is involved in many (patho)physiological processes in several tissues such as the central nervous system (CNS) [3, 4] in which it has been considered as a neuromodulator molecule [5], playing a critical role in the control of neuronal dysfunction and neurodegeneration [6]. In fact, our group reported that adenosine A_1 and A_{2A} receptors are altered not only in Alzheimer's disease [7], Parkinson's disease [8], Huntington's disease [9], Pick's disease [10, 11] and schizophrenia [8] but also in the brain from very young senescence accelerated mouse-prone 8 (SAMP8) mice [12]. This mouse model is considered as an aging and Alzheimer's disease model due to its ability to mimic some phenotypic hallmarks in every stage of this disease such as overexpression of amyloid-β (Aβ), high levels on the gene expression of

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presenilin-2 and Tau in the hippocampus, but a lower expression of apolipoprotein-E when compared to control [13]. Changes on adenosine receptors level in SAMP8 versus SAMR1 resistant strain were detected in whole brain by our group [12]. Although it remains to elucidate whether SAMP8 mice reproduce regional specific changes on adenosinergic system reported to date in AD patients [2, 7, 14–16], the main objective in this study was to analyse the possible modulation of adenosine receptors by resveratrol in SAMP8 mice.

Resveratrol (RSV) is a natural polyphenol present in berries, peanuts, grape skin and red wine that has showed many beneficial properties for health since it was discovered to have a therapeutic role in vivo years ago [17], including its neuroprotective role on neurodegeneration [18]. It seems that neuroprotective action of this compound is mainly due to its ability to attenuate neuroinflammation [19–23], oxidative stress [24] and Aβ clearance [25] and aggregation [26]. Recently, some authors have reported that RSV could act as a phytoestrogenic agonist [27], and this might stimulate adenylyl cyclase in an independent manner of estrogen receptor activation [28]. It has been reported that RSV exhibits an inhibitory role on the phosphodiesterase IV activity [29], which is in line with its reported effect of increase cAMP levels and AMPK activation [30]. RSV action on adenosinergic system is not being intensely investigated. It was proposed in oculohypertensive rats that resveratrol could interact with adenosine receptors, triggering an agonistic role via adenosine A_1 receptor [31]. In addition, RSV showed an antiepileptic action in an adenosinergic system-dependent manner as a significant reduction in the percentage of incident generalized tonic-clonic convulsions was observed when adenosine was administered together with RSV, whereas the nonspecific adenosine receptor antagonist theophylline significantly reversed the RSV-induced protection [32]. However, the molecular mechanism by which RSV affects to adenosine signalling pathways still remains unknown.

In spite of the controversy about the possible effects of RSV due to its rapid metabolism into other metabolites, there is a growing body of evidence about the therapeutic potential in vivo of RSV [17]. Results we show herein clearly demonstrate that this polyphenol modulates the adenosine-mediated signalling pathways, suggesting a protective role by resensitizing A_1R-mediated signalling and desensitizing A_{2A}R-mediated signalling in RSV-treated mice when compared to untreated mice.

Materials and Methods

Animals and RSV Diet

A total of 26 male SAMP8 mice from 5 to 7 months old (mo) were used for this study. Mice received a standard diet (2018 Teklad global 18% protein rodent maintenance diet, Harlan) or the same diet supplemented with transresveratrol (1 g/kg, having a diary dose of 160 mg/kg, Mega Resveratrol, Candlewood Stars, Inc., CT, USA), starting from the weaning or 4 mo for 5 and 7 mo mice, respectively, to 1 month before sacrifice day. All the mice had food and water ad libitum and were kept in standard conditions of temperature (22 ± 2 °C) and 12:12-h light–dark cycles (300/0 lx). Studies were performed in accordance with the institutional guidelines for the care and use of laboratory animals established by the Ethical Committee for Animal Experimentation at the University of Barcelona.

Sample Preparations from SAMP8 Mice

Plasma membranes from SAMP8 mice brain were isolated as previously described [33]. Whole brain preparations (excluding cerebellum and spinal cord) were homogenized in 20 volumes of isolation buffer (50 mM Tris–HCl, pH 7.4, containing 10 mM $MgCl_2$ and protease inhibitors) in Dounce homogenizer (10xA, 10xB). After homogenization, samples were centrifuged for 5 min at 1.000× g in a Beckman JA 21 centrifuge. Supernatants were centrifuged again for 20 min at 27.000× g, and the resulting supernatant was considered as cytoplasmic fraction, and pellet (plasma membranes) was resuspended in isolation buffer. Whole brain homogenate (200 μL) from each sample was stored at − 80 °C for total RNA isolation. Protein levels were measured by Lowry method.

Total RNA Isolation and cDNA Preparation

Total RNA was extracted from whole brain homogenate (excluding cerebellum and spinal cord) using an ABI 6100 Nucleic Acid PrepStation according to the manufacturer's protocol (P/N 4330252 rev C). Briefly, homogenate was incubated in nucleic acid purification lysis solution (P/N 4305895) and then purified by passing the lysate through a purification tray containing an application-specific membrane (P/N 4305673). Wash solutions were applied to the membrane, and the purified RNA was eluted into a 96-well PCR plate. DNase treatment using AbsoluteRNA Wash Solution (P/N 4305545) was included during purification to remove genomic DNA from samples. All chemicals for the ABI 6100 were purchased from Applied Biosystems (Madrid, Spain). Total RNA isolated from mice was stored at − 80 °C. Ratio of A_{260}/A_{280} (purity of RNA) was in the range 1.7–2.3. RNA concentrations were determined from the A_{260}. One microgram of total RNA was reverse transcribed using Applied Biosystems High-Capacity cDNA Archive Kit (P/N 4368813).

Quantification of Gene Expression by Real-Time PCR

Quantitative real-time RT-PCR analysis was performed with an Applied Biosystems Prism 7500 Fast Sequence Detection

System using TaqMan universal PCR master mix according to the manufacturer's specifications (Applied Biosystems Inc., Madrid, Spain). The validated TaqMan probes and primers for A_1R (assay ID Mm01308023_m1), $A_{2A}R$ (assay ID Mm00802075_m1), $A_{2B}R$ (assay ID Mm00839292_m1), A_3R (assay ID Mm01296602_m1) and β-actin (assay ID Mm00607939_s1) were assay-on demand gene expression products from Applied Biosystems. The TaqMan primer and probe sequences are packaged together in a 20× solution. Mouse β-actin gene was used as endogenous control. Gene expression assay was carried out by following the manufacturer's indications as described [34]. The thermal cycler conditions were as follows: hold for 20 s at 95 °C, followed by two-step PCR for 40 cycles of 95 °C for 3 s, followed by 60 °C for 30 s. Levels of RNA expression were determined using the 7500 Fast System SDS software version 1.3.1 (Applied Biosystems) according to the $2^{-\Delta\Delta Ct}$ method. Briefly, expression results for a gene were normalized to internal control actin relative to a calibrator, consisting of the mean expression level of the corresponding gene in control samples as follows: $2^{-\Delta\Delta Ct} = 2^{-(Ct\ \text{receptor gene}-Ct\ \text{actin gene})\ \text{sample}-(Ct\ \text{receptor gene}-Ct\ \text{actin gene})\ \text{calibrator}}$. Assays were performed in duplicated on different plates each using different cDNAs from the animals analysed. Results were averaged to produce a single mean quantity value for each mRNA for each animal.

Radioligand Binding Assays in Plasma Membrane

Binding assays to plasma membranes from SAMP8 mice brain were performed as described previously [33]. Plasma membranes were incubated with 5 U/mg adenosine deaminase (ADA) in 50 mM Tris–HCl, 2 mM $MgCl_2$, pH 7.4, for 30 min at 25 °C, in order to remove endogenous adenosine from samples. Then, 50 μg of plasma membranes was incubated with 20 nM [^{3}H]DPCPX or 20 nM [^{3}H]ZM 241385 for 2 h at 25 °C as previously reported [12]. In order to obtain nonspecific binding, 1 mM CPA or 3 mM theophylline was used as displacing ligand for A_1R and $A_{2A}R$, respectively. Binding assays were stopped by rapid filtration through Whatman GF/B filters, previously pre-incubated with 0.3% polyethylenimine using a FilterMate Harvester (PerkinElmer). Scintillation liquid mixture was added in order to measure radioactivity in a Microbeta Trilux (PerkinElmer) liquid scintillation counter.

Western Blotting Analysis

For Western blotting assays, 50 μg of membrane protein and 15 μg of cytoplasm protein were mixed with loading buffer containing 0.125 M Tris (pH 6.8), 20% glycerol, 10% β-mercaptoethanol, 4% SDS and 0.002% bromophenol blue, and heated at 95 °C for 5 min. Protein was electrophoresed on a 10% SDS–PAGE gel using a Mini-Protean system (Bio-Rad) with molecular weight standards (Bio-Rad, Madrid, Spain). Protein transfer to nitrocellulose membranes was carried out in iBlot™ Dry Blotting System (Invitrogen, Madrid, Spain). Membranes were washed with PBS-Tween 20, blocked with PBS containing 5% skimmed milk and then incubated with the primary antibodies at 4 °C overnight (1:500 dilution for anti-$A_{2B}R$; 1:1000 dilution for anti-A_3R, anti-AC, anti-synapsin, anti-GFAP and anti-PKA; and 1:5000 dilution for anti-β-actin). After rinsing, the membranes were incubated with the corresponding secondary antibody (Bio-Rad, Madrid) at a dilution of 1:5000 in PBS containing 5% skimmed milk for 30 min. Antigen was visualized using the ECL chemiluminescence detection kit (Amersham, Madrid, Spain), and specific bands were quantified by densitometry using GeneTools (Bio-Rad).

Adenylyl Cyclase Activity Determination

Adenylyl cyclase activity was determined in brain plasma membranes as previously described [35]. Assays were performed with 10–20 μg of protein in a final volume of 0.25 mL of 50 mM Tris–HCl, pH 7.4, 5 mM $MgCl_2$, 1 mM 1,4-dithiothreitol, 1 mg/mL bovine serum albumin, 1 mg/mL creatine kinase, 10 mM creatine phosphate and 0.1 mM Ro 20-1724 (a specific phosphodiesterase inhibitor). Plasma membranes, previously incubated with adenosine deaminase (2 U/mg protein) at 37 °C for 30 min to remove endogenous adenosine, were incubated for 15 min at 37 °C in the absence or the presence of 5 μM forskolin, 100 μM CHA and 100 μM CGS 21680 followed by a further incubation with 2 mM ATP at 30 °C for 10 min. Reaction was stopped by the boiling of samples and then centrifugation at 12.000×*g* for 4 min. Fifty microlitres of supernatant was used to determine cAMP accumulation. Samples were incubated with 0.25 pmol of [^{3}H]cAMP and 6.25 μg protein kinase A in a final volume of 200 μL of buffer assay (50 mM Tris–HCl, pH 7.4, 4 mM EDTA) for 2 h at 4 °C. Standard samples were prepared in the same buffer in the range 0–32 pmol of cAMP. Reaction was stopped by boiling the samples at 100 °C for 5 min and then centrifuged at 12.000×*g* for 4 min. Radioactivity was measured in a Microbeta Trilux (PerkinElmer) liquid scintillation counter.

5′-Nucleotidase Activity Assay

About 20 lg of cerebral cortical membranes was pre-incubated in 180 lL of the reaction medium containing 50 mM Tris, $MgCl_2$ 5 mM, pH 9, at 37 °C for 10 min. Then, the reaction was initiated by the addition of 20 lL AMP (final concentrations, 1 lM–3 mM) and stopped 10 min later by adding 200 lL of 10% trichloroacetic acid. The samples were chilled on ice for 10 min and then centrifuged at 12,000×*g* for 4 min at 4 °C. The supernatants were used to measure inorganic phosphate released following the protocol described by Chan et al.

(1986) using KH_2PO_4 as Pi standard. Nonenzymatic hydrolysis of AMP was corrected by adding cerebral cortical membranes after trichloroacetic acid.

Incubation times and protein concentration were selected in order to ensure the linearity of the reactions. All samples were run in triplicate. Enzyme activity is expressed as nmol Pi released/min/mg of protein.

Plasma membrane (30 μg protein) and cytosolic fraction (30 μg protein) from SAMP8 brain (excluding cerebellum and spinal cord) were pre-incubated in reaction medium (50 mM Tris–HCl, 5 mM $MgCl_2$ pH 9), at 37 °C for 10 min. Then, the reaction was initiated by adding AMP at final concentration 500 μM and stopped 20 min later by adding 10% trichloroacetic acid. The samples were chilled on ice for 10 min and then centrifuged at 12,000× *g* for 4 min at 4 °C. The supernatants were used to measure inorganic phosphate released as previously reported [36] using KH_2PO_4 as Pi standard. Nonenzymatic hydrolysis of AMP was corrected by adding samples after trichloroacetic acid. Incubation times and protein concentration were selected in order to ensure the linearity of the reactions. All samples were run in duplicate. Enzymatic activity is expressed as nmol Pi released/min mg protein.

Adenosine Deaminase Activity Assay

Enzyme activity assay kit (Abcam ab204695) was performed according to the manufacturer's protocol (Abcam, Cambridge, UK). Cytoplasm protein was diluted 1:100 in ADA Buffer Assay in 96-well plate and assayed in duplicated. Then, 96-well plate was read at Ex/Em = 535/587 nm as kinetic curve for 30 min. Sample values were obtained by interpolation in an inosine standard curve performed in parallel in the same plate as previously described [2].

Purine Level Quantification by HPLC

Chromatographic analysis was performed with Ultimate 3000U-HPLC, and data peaks were processed with Chromaleon 7 (ThermoFisher, Madrid, Spain) as previously described [2]. HPLC diode array was used working at 254-nm wavelength. Purine standards and samples (40 μL) were injected in C18 column of 4.6 mm × 250 mm, 5-μm particle size. Two solvents were used for gradient elution: solvent A 20 mM phosphate-buffered solution (pH 5.7) and solvent B 100% methanol. The gradient was 95% (11 min), 80% (9 min) and 95% (2 min) in solvent A. The total run time was 22 min with a constant flow rate of 0.8 mL/min at 25 °C. Retention times for hypoxanthine, xanthine, inosine, guanosine and adenosine were 3.5, 3.9, 8.4, 9.4 and 15.5 min, respectively. Each purine level was obtained by interpolation from the corresponding purine standard curve. The standard curves were obtained by using five concentrations of each purine ranging from 0.1 to 500 μM. Data were then normalized to the protein concentration of each sample.

Statistical and Data Analysis

Data are means ± SEM. Statistical analysis was according to Student's *t* test. Differences between mean values were considered statistically significant at $p < 0.05$. Correlations were analysed by Pearson correlation. GraphPad Prism 6.0 programme was used for statistical and data analysis (GraphPad Software, San Diego, CA, USA).

Results

RSV Effect on the Body Weight Gain

RSV treatment was carried out through an oral administration as previously described in "Materials and Methods" section. Body weight gain was not affected by RSV for 5 mo mice as represented in Fig. 1a. However, in 7 mo mice, RSV seems to have a slight tendency to maintain body weight when compared to untreated mice of the same age. Moreover, as we can see in Fig. 1b, this tendency appears to be significant from the fifth week.

RSV Effect on Adenosine Receptor (ADORA) Gene Expression

After total RNA isolation, real-time PCR assay was performed in order to know whether RSV treatment in vivo causes any effect on adenosine receptor gene expression. If we first carefully check the age-related effect on the all four adenosine receptors, we can observe a significant downregulation in the A_1, A_{2A} and A_{2B}, while the opposite effect in A_3 receptor was detected (Fig. 2). Moreover, we found that RSV differently modulates adenosine receptor gene expression when compared to untreated mice. Concerning to A_1 and A_{2A} receptors, lower mRNA levels were detected in treated mice when compared to 5 mo untreated mice, while at 7 mo, no changes on gene expression were observed (Fig. 2a, b). A_{2B} mRNA levels were not altered by RSV treatment (Fig. 2c). Nevertheless, A_3R gene expression was altered in a different way after RSV treatment, while in 5 mo mice, an upregulation was detected; lower mRNA levels were obtained in 7 mo mice (Fig. 2d).

RSV Effect on Adenosine Receptor Modulation

In order to know whether RSV was able to affect the adenosine receptor presence on plasma membrane as suggested by gene expression assays, we carried out two different techniques to quantify these receptors. Figure 3 shows the

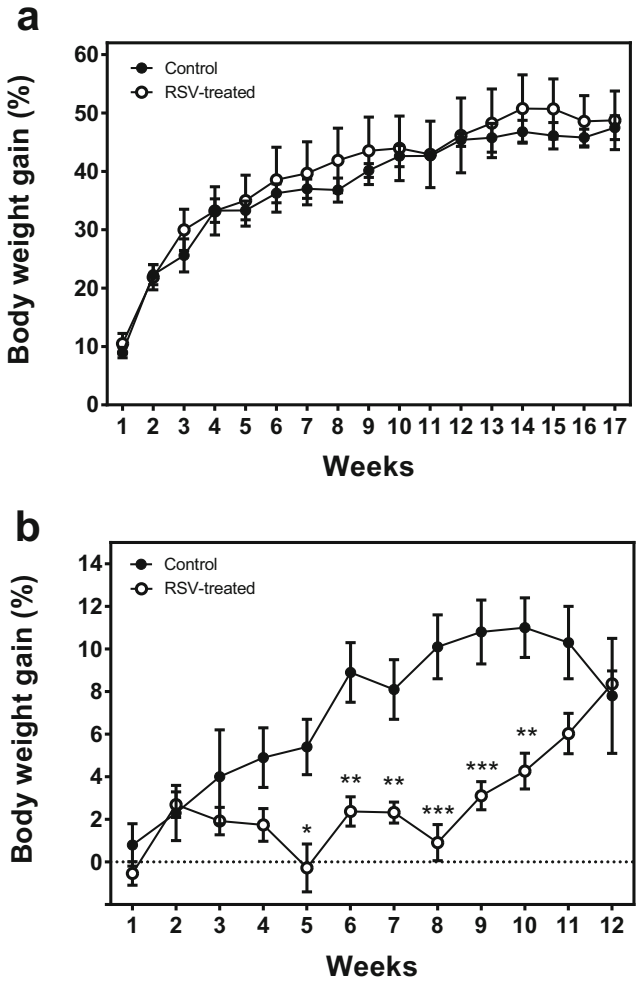


Fig. 1 Effect of RSV on the body weight gain. Animals were supplemented with RSV as described in "Materials and Methods" section. Body weight gain was measured every week during the entire treatment, and results are represented in the graphs as percentage of gain with respect to the body weight in the 5-month-old group (**a**) and 7-month-old group (**b**). Data are the mean ± SEM from 59 different samples. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ significantly different from control mice according to Student's *t* test

modulation of adenosine receptor as detected by radioligand binding assay. A_1 receptors appear to be significantly reduced in an age-related process (Fig. 3a) which is in line with the results obtained in the gene expression analysis (Fig. 1a). No changes associated with age were detected in the case of A_{2A} receptors (Fig. 3b). However, RSV treatment was able to cause a significant upregulation of A_1 in both 5 and 7 mo mice, being strongly upregulated at 7 mo mice (Fig. 3a). This modulation may suggest a reversing effect caused by the significant age-related downregulation of this receptor. On the other side, A_{2A} receptors were not affected by the RSV in any case (Fig. 3b), suggesting that RSV intake might affect mainly through A_1R-mediated signalling.

In addition, as shown in Figs. 4 and 5, it was detected a modulation of A_{2B} and A_3 receptors on the plasma membrane fraction, respectively. In the case of A_{2B}, it seems to be increased during aging (Fig. 4a). Yet, RSV caused a significant increase in A_{2B} receptor level at 5 mo mice (Fig. 4b), with no changes observed in 7 mo mice when compared to their corresponding untreated mice (Fig. 4c). On the other side, A_3 receptor seemed to be unaltered by age (Fig. 5a). RSV only caused a significant A_3 decrease in 7 mo mice when compared to their corresponding untreated mice (Fig. 5c), with no effect in 5 mo mice (Fig. 5b).

RSV Effect on the A_1 and A_{2A}-Mediated Signalling Pathways

As adenosine receptor modulation was detected, we next performed the analysis of signalling pathways mediated by A_1 and A_{2A} because they are the main adenosine receptors expressed in the brain. As shown in Fig. 6, untreated mice showed a clear loss of the A_1-mediated signalling pathway in both 5 and 7 mo mice (Fig. 6b), which is in line with the decrease in gene expression and protein levels detected. Moreover, RSV induced a clear A_1-mediated signalling resensitization in both 5 and 7 mo mice (Fig. 6b), suggesting that RSV action might avoid the loss of A_1 functionality. However, A_{2A}-mediated signalling pathway appeared to be completely functional in both 5 and 7 mo untreated mice (Fig. 6c), but in this case, RSV caused a clear A_{2A}-mediated signalling desensitization. In addition, basal activity of adenylyl cyclase was not altered either during aging or after RSV treatment (Fig. 6a).

RSV Effect on 5′-Nucleotidase and Adenosine Deaminase Activities

Next, we aimed to know whether RSV was able to modulate the activities of these two enzymes, which are involved in the control of adenosine levels. Figure 7 shows a clear modulation in both enzymes by RSV treatment. An inhibitory effect on the 5′-nucleotidase activity from plasma membrane in 5 mo-treated mice was detected when compared to their corresponding untreated mice (Fig. 7a). Yet, it was also observed a significant age-related loss of this enzyme activity (Fig. 7a). In addition, 5′-nucleotidase activity from cytoplasm fraction was significantly reduced in 7 mo treated when compared to their corresponding untreated mice (Fig. 7b). In the same way, adenosine deaminase (ADA) activity was also reduced by RSV treatment with a stronger effect on younger mice (Fig. 7c). However, no age-related effect on ADA activity was observed, suggesting that the stronger inhibitory effect observed in younger treated mice might be due to the RSV supplementation from weaning. According to these results, we suspected that adenosine levels might be altered by RSV.

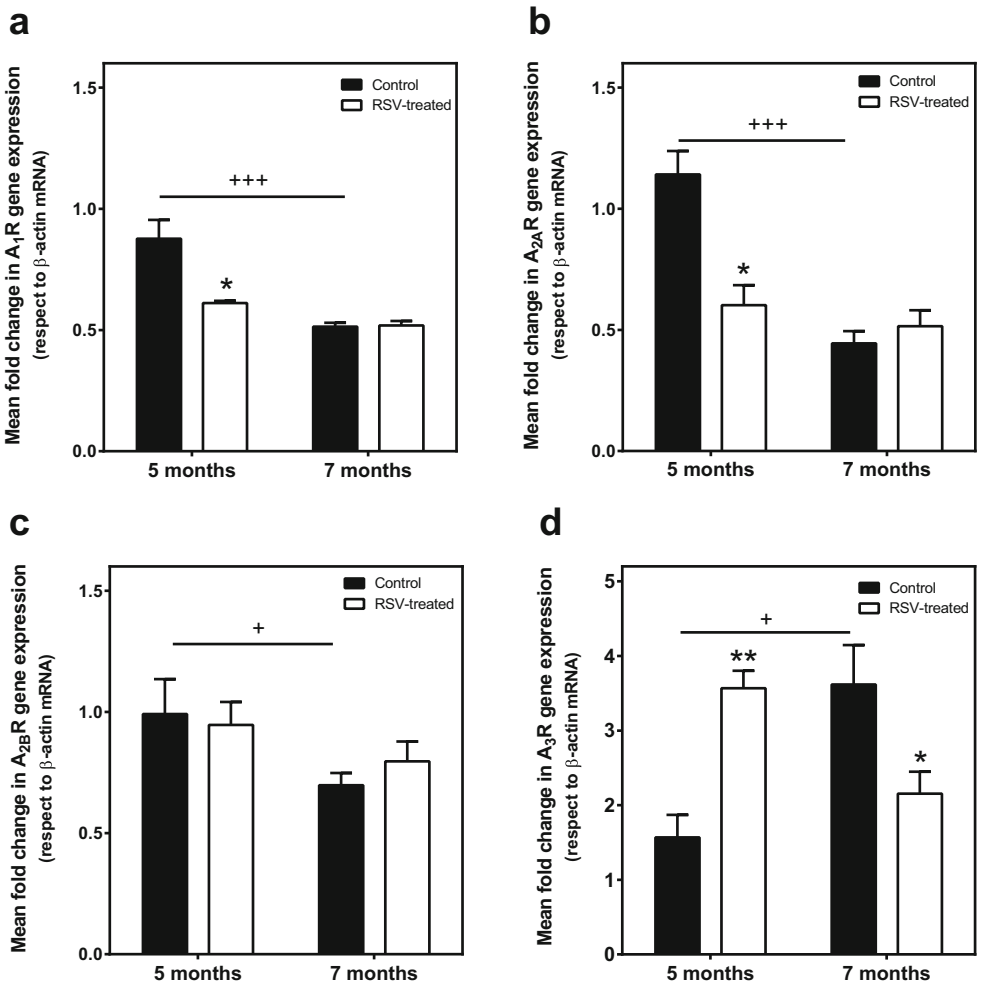


Fig. 2 Adenosine receptor gene expression assayed by quantitative real-time RT-PCR. After total RNA isolation, A_1R (**a**), $A_{2A}R$ (**b**), $A_{2B}R$ (**c**), and A_3R (**d**) gene expression levels were detected by using TaqMan universal PCR following the protocol indicated in "Materials and Methods" section. β-Actin was used as an endogenous control in all assays. Data show the mean ± SEM of 59 different samples. $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$ significantly different from control mice and $+p < 0.05$ and $+++p < 0.001$ significantly different from 5-month-old mice, all according to Student's *t* test

Adenosine and Related Metabolite Levels

Once we observed some differences on enzymatic activities related to adenosine metabolism, we next tried to detect the adenosine and related metabolite level by using HPLC as described in "Materials and Methods" section. Figure 8 represents all measured purines, showing that adenosine and its metabolites seem to be significantly reduced in an age-related manner. RSV caused a lower level only in adenosine and inosine in 5 mo mice. However, at 7 months, RSV reduced xanthine levels and increased inosine and guanosine levels, while hypoxanthine and adenosine remained unchanged.

Correlation Analysis Within Adenosinergic System

To investigate the effect of RSV action on the adenosine-mediated signalling, all components from adenosinergic system, even the functionality of adenosine A_1 and A_{2A} receptors, were correlated with each other. As observed in the supplemental figs. (SF), it appears to exist a higher correlation level in 5 mo mice than that detected in 7 mo mice. According to this, in the case of 5 mo mice (SF 1), the A_1 and A_{2A}-mediated signalling pathways showed a negative correlation, which means that the more A_{2A}-mediated signalling, the less A_1 functionality. Moreover, the 5′-nucleotidase activity seems to have a negative correlation with both A_1 and A_{2A}, that is, the more enzymatic activity, the less presence on plasma membrane of the corresponding receptor. Yet, ADA and 5′-nucleotidase activities also correlated positively; even both enzymes showed a positive correlation with adenosine level. Nevertheless, in the case of 7 mo mice (SF 2), only a significant negative correlation was observed between A_{2A} and A_1 functionality, which strongly suggests that older adenosinergic system loose the higher correlation detected in younger mice indicating a possible deregulation of adenosinergic system with age.

Discussion

Results shown herein clearly demonstrate that resveratrol is able to modulate adenosine-mediated signalling in SAMP8 mice. This polyphenol caused a desensitization of A_{2A}-

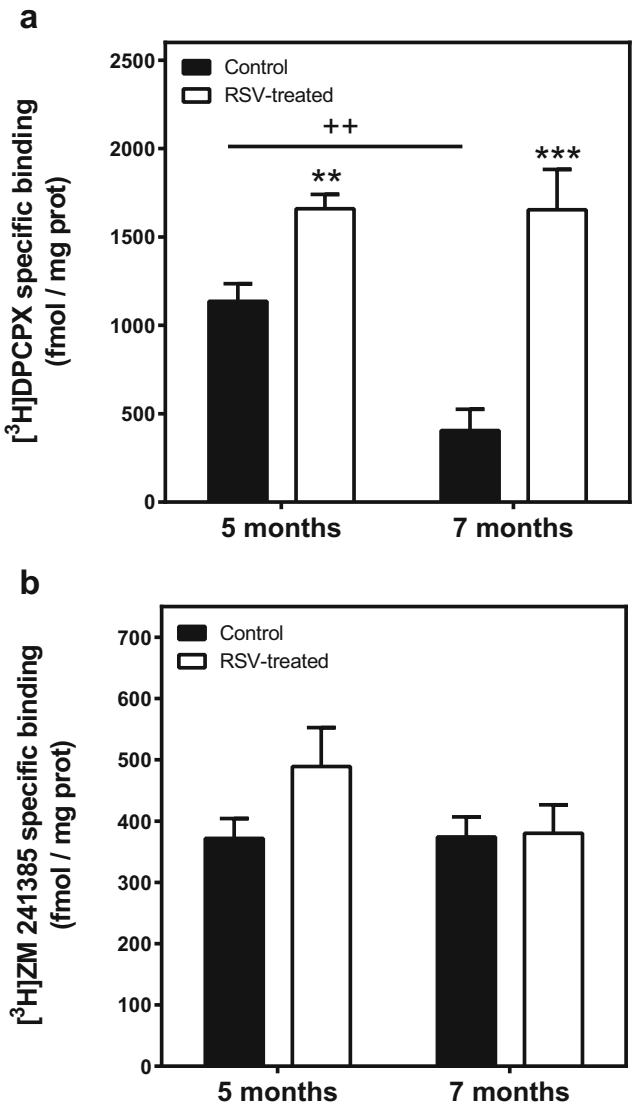


Fig. 3 Analysis of adenosine A_1 and A_{2A} receptors by radioligand binding assay. Specific binding to plasma membrane was determined at saturation concentration (20 nM) of [^{3}H]DPCPX for A_1R (**a**) and [^{3}H]ZM 241385 for A_{2A}R (**b**) as described in "Materials and Methods" section. Nonspecific binding was determined in the presence of 1 mM CPA and 3 mM theophylline for A_1R and A_{2A}R, respectively. Data show the means ± SEM of 59 different samples. $^{**}p < 0.01$ and $^{***}p < 0.001$ significantly different from control mice and $^{++}p < 0.01$ significantly different from 5-month-old mice, all according to Student's *t* test

mediated signalling, and it was also able to avoid the age-related loss of A_1, which is in line with the re-sensitization of A_1-mediated signalling observed in RSV-treated mice.

Since it has been discovered that RSV exerts different biological actions, including a neuroprotective role, many in vivo studies and clinical trials have been carried out in order to elucidate whether RSV is able to attenuate the cognitive decline in neurodegenerative diseases. Some data from in vivo studies demonstrated that this polyphenol improved spatial memory in murine model [37, 38], even in SAMP8 mice [39]. RSV also appears to prevent this cognitive decline [40]

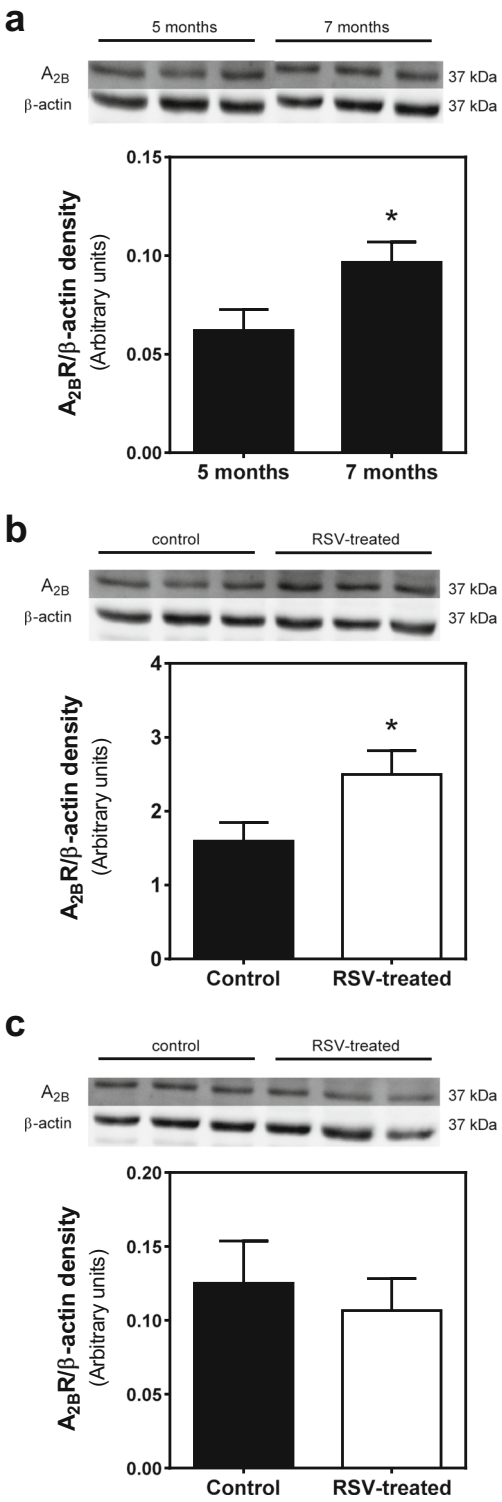
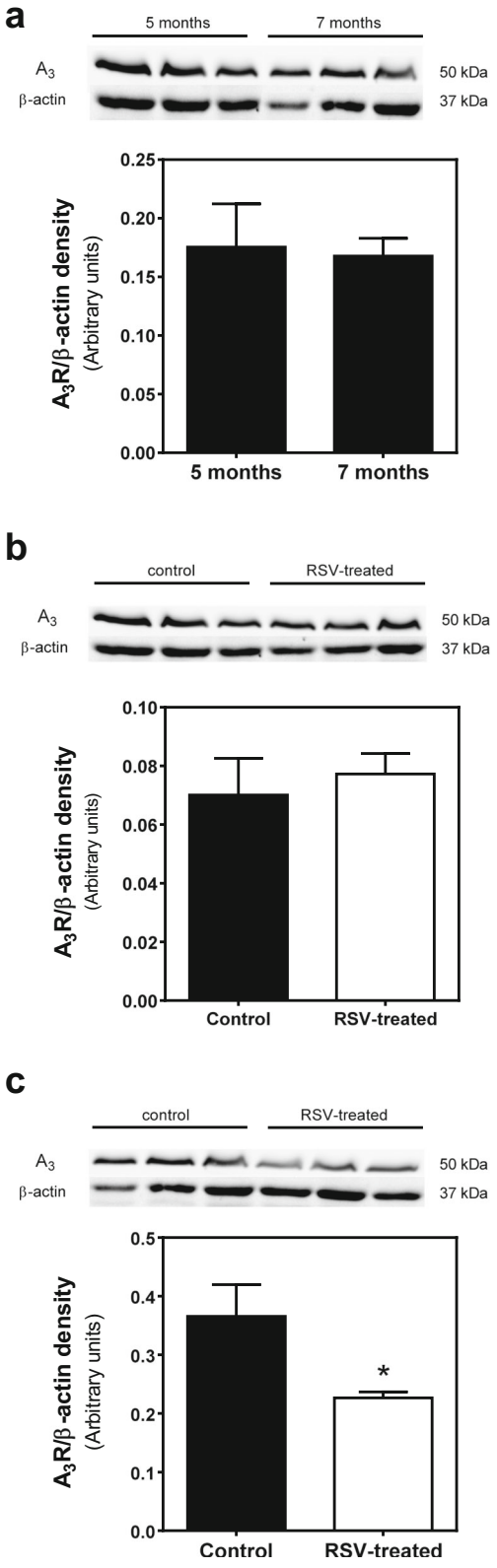


Fig. 4 Detection of adenosine A_{2B} receptor from plasma membrane fraction. A_{2B}R detection was carried out by Western blotting assay in plasma membrane fraction as described in the previous section. Graphs represent the modulation of this receptor during aging (**a**) and after RSV treatment in 5- (**b**) and 7-month-old mice (**c**). Data are means ± SEM after densitometric analysis of the corresponding bands of 56 different samples related to β-actin value which was used as loading control. $^{*}p < 0.05$ significantly different according to Student's *t* test



◀ **Fig. 5** Detection of adenosine A_3 receptor from plasma membrane fraction. A3R detection was carried out by Western blotting assay in plasma membrane fraction as described in the previous section. The figure shows the modulation of this receptor during aging (**a**) and after RSV treatment in 5- (**b**) and 7-month-old mice (**c**). Data are means ± SEM after densitometric analysis of the corresponding bands of 56 different samples related to β-actin value which was used as control loading. $*p < 0.05$ significantly different according to Student's *t* test

and extend the life span when compared to controls [41]. We have previously published behaviour changes (including cognitive and emotional parameters) in SAMP8 mice. Different behaviour and learning parameters in SAMP8 and SAMR1 are impaired by age and related with oxidative stress environment [42]. This work presented an impairment in emotional behaviour with regard to fear and anxiety in young SAMP8 vs age-matched SAMR1. In contrast, learning capabilities are worse in SAMP8, both in young and aged animals, with regard to SAMR1. These waves in behaviour and cognition correlated with an excess of oxidative stress in SAMP8 at younger ages that diminished with age. Now, in the present work, we show changes in adenosine receptors and adenosinergic system gated to neuronal markers of cell function in the same model; then, it could be said that changes in adenosinergic system are in part cause or consequence for cognitive impairments in SAMP8; indeed, the two parameters (adenosinergic system and cognition) are modulated by RSV treatment. The neuroprotective role of RSV and its impact on cognition have been also demonstrated in SAMP8. Long-term RSV treatment significantly prevented memory loss as measured by the object recognition test. Moreover, RSV reduced the amyloid burden and increased mitochondrial complex IV protein levels in mouse brain [41]. Results presented in the present work represent the first causative approach which demonstrates a role for adenosinergic system in the senescence, early AD hallmarks and cognitive decline in this mouse strain. Currently, several clinical trials in humans have been completed with no conclusive evidence about beneficial properties of RSV about cognitive functions, as described elsewhere [43]. These nonsatisfactory outcomes obtained from the different trials might be perhaps explained by the RSV dose and duration of treatment. One of the clinical trials that should be taken into account due to its big amount of individuals analysed from mild to moderate Alzheimer's disease, the long term and the high dose of RSV employed, is the one carried out by Turner and colleagues [44]. They reported that the decline of level of $A\beta_{1–40}$ and $A\beta_{1–42}$ in CSF and plasma with age detected in placebo group was reduced in RSV-treated group, which might be attributed to the ability of RSV to modulate Aβ aggregation and clearance as reported in different cell lines expressing wild-type Aβ [25, 26]. Thus, the lower level of these toxic peptides observed in CSF and plasma from placebo group may be due to the anti-Aβ aggregation effect of RSV in RSV-treated individuals. On the other hand, the no improvement on cognition in these patients may suggest that maybe it is "too late" to reverse the memory loss due to the massive neuronal death in early stages of AD patients. In this study [44], authors reported a body weight loss

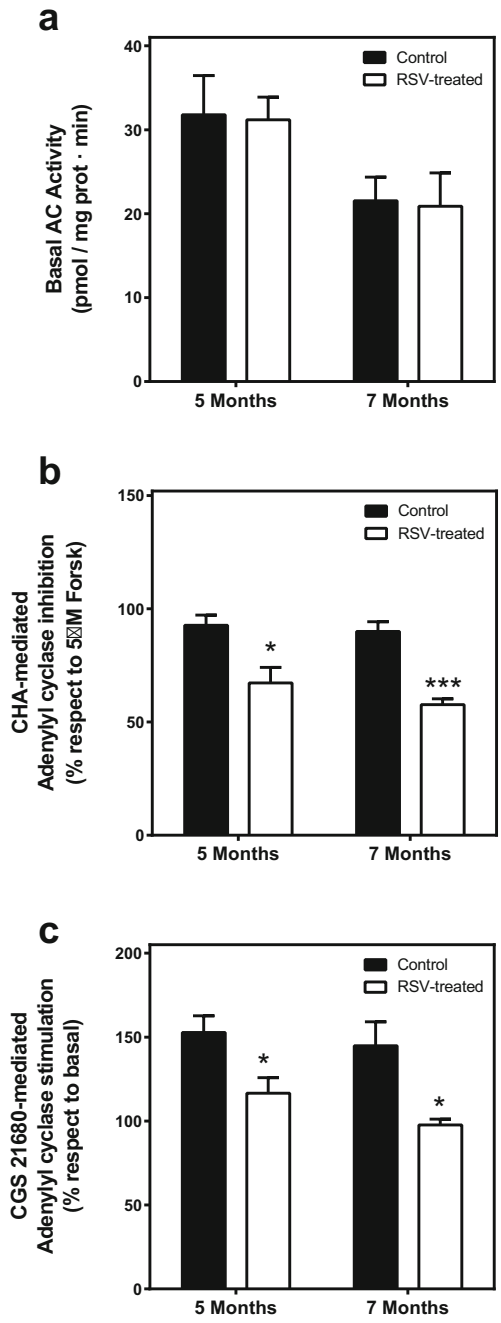


Fig. 6 Effect of RSV treatment on adenylyl cyclase activity. Plasma membranes were used to analyse the adenylyl cyclase activity as described in "Materials and Methods" section. **a** The basal activity of the enzyme from both mice groups and represented as pmol/mg protein min. **b** The A_1R-mediated signalling by using CHA, a potent agonist for A_1R, and represented as percentage respect to 5 μM forskolin stimulation. **c** The A_{2A}R-mediated signalling pathway by using CGS 21680, a selective agonist for A_{2A}R, and represented as percentage respect to basal activity of the enzyme. Data are the mean ± SEM of 59 different samples. $^*p < 0.05$ and $^{***}p < 0.001$ significantly different from their corresponding control according to Student's *t* test

in patients with RSV treatment which could be due to an increased mitochondrial activity after RSV action. Resveratrol modulates mitochondrial function and dynamics by diverse mechanisms, causing cytoprotective effects in both in vitro and in vivo experimental models involving brain cells [45–47]. However, it has been reported no difference for final body weight among control SAMR1, control SAMP8 and RSV-treated SAMP8 mice groups at the end of treatment (i.e. 24 weeks) [48]. RSV has been reported to reduce fat mass in obese but not lean mice [49] which may be due to inhibition of pre-adipocyte proliferation, and inhibition of adipogenic differentiation [50]. Interestingly, adenosine A_1, A_{2A} and A_{2B} have an impact on adipocyte function, and overexpression of A_1 receptors in adipose tissue protects mice from obesity-related insulin resistance [51]. Keeping in mind that RSV-induced effects on body fat differ depending on feeding conditions [52] and that some metabolic differences may exist between animal models and humans [53], we can hypothesize that differences in body weight gain detected in the present study in 7 mo mice could reflect changes in A_1, A_{2B} and A_3 levels, which would merit further research.

Molecular mechanism of RSV action is not very well understood, but many authors bet for anti-inflammatory and antioxidant properties of this molecule together with its ability of antiaggregation of Aβ [54–56], as previously detailed. Little is known about the role of RSV on adenosinergic system. Our results indicate that gene expression of all four adenosine receptors shows a different tendency when compared to the protein level of the receptor presence in plasma membrane. Lack of full correspondence between mRNA and protein levels is not unusual, and post-transcriptional processes may lead to stronger deviations from an ideal correlation [57]. Similarly, previous data showed that gene expression of adenosine receptors was not associated with receptor protein levels expressed in plasma membrane in the frontal cortex in Alzheimer's disease [7], which may suggest a post-transcriptional regulatory process of adenosine receptors. On the other hand, some studies suggest that this phytochemical could interact with adenosine receptors [31, 58]. Therefore, the modulation observed on adenosine receptors by RSV treatment could be due to that direct interaction. According to this, our results also seem to indicate an agonistic effect through A_1 receptor. Perhaps this agonistic effect and the re-sensitization of the A_1-mediated signalling pathway after RSV treatment might be caused by avoiding the age-related loss of A_1 observed in this model [12]. This age-related decrease in the distribution of A_1 receptors has also been reported in the thalamus and frontal, temporal, occipital and parietal cortices from healthy human volunteers [59], in the hippocampus, cortex, basal ganglia and thalamus from aged mouse [60] and in the hippocampus, thalamus and some cortical and septal regions from rat brain [61]. The widespread reduction in A_1 receptor abundance in brain areas where adenosine plays a significant neuromodulatory role must have functional consequences [62]. Other peripheral systems as cardiac function can also be affected. Thus, the presynaptic A_1-mediated inhibition

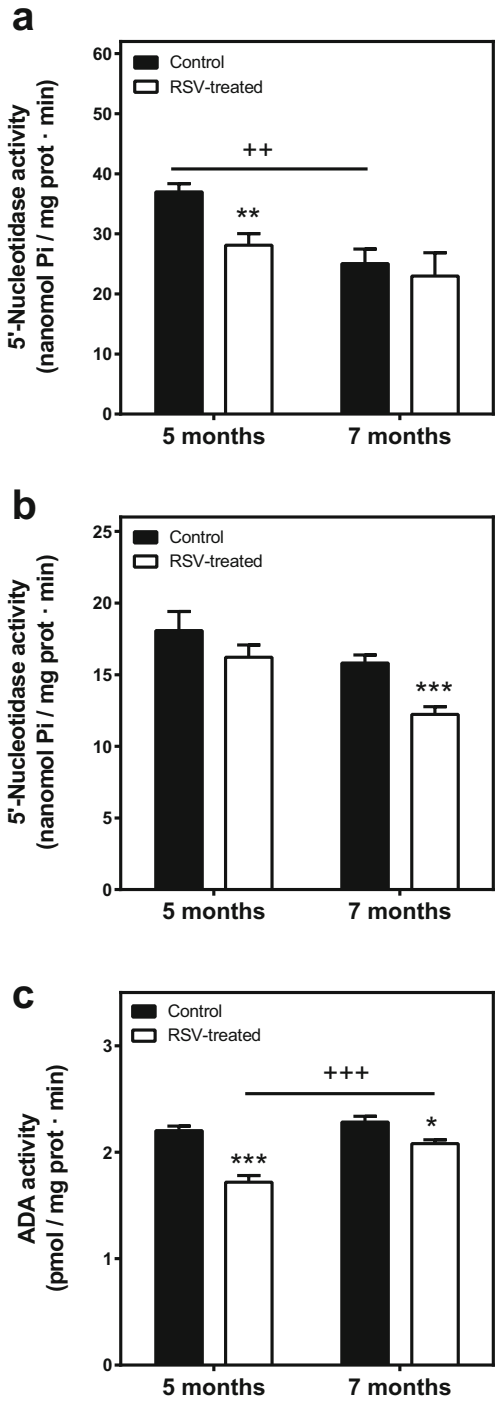


Fig. 7 Effect of RSV treatment on adenosine-related enzymatic activities. 5′-Nucleotidase activity from plasma membrane (**a**) and cytosol fraction (**b**) was measured as described in "Materials and Methods" section. Results are represented as nmol of Pi released/mg protein min. Adenosine deaminase activity (**c**) was performed by following the manufacturer's protocol, and results are expressed as pmol/mg protein min. Data are the mean ± SEM of 59 different samples. $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$ significantly different from their corresponding control and $++p < 0.01$ and $+++p < 0.001$ significantly different from 5-month-old mice, all according to Student's *t* test

of norepinephrine release in the rat heart declines with age [63], and the ischemic tolerance changes in aged mouse hearts involving transcriptional regulation of all adenosine receptors [64]. In addition, the desensitization of A_{2A}-mediated signalling together with the agonistic effect observed in A_1 functionality in our data might suggest some neuroprotective properties of this molecule on cognitive decline via adenosine signalling. Indeed, our group reported a strong downregulation of A_1 as a very early event during aging from SAMP8 mice in which 21-day old mice had a lower A_1 expression in brain as compared to SAMR1 mice strain, while A_{2A} were found to be significantly increased in older mice [12]. Yet, adenosine receptors are also increased in neurodegenerative diseases such as Alzheimer's [7], Parkinson's [8], Huntington's [9], schizophrenia [8] and Pick's disease [10, 11]; even its endogenous ligand, adenosine and their converting enzymes appear to be altered in Alzheimer's [2] and Parkinson disease [65].

Our data strongly suggest that RSV treatment caused either desensitization or blockade of A_{2A}-mediated signalling, while A_1 receptor functionality was increased compared to control mice. These results could explain, at least partially, some of the beneficial properties of this polyphenol in neurodegeneration. In fact, it has been postulated that A_{2A} plays a relevant role in neuronal dysfunction [6], synaptic deficits [66] and memory impairment [67]. In line with this, several authors have reported that caffeine intake [68, 69], a natural antagonist for this receptor, or even pharmacological blockade of A_{2A} [70, 71] improves cognition and memory, and prevents from Aβ accumulation [69, 72] and its corresponding mediated toxicity [71]. Moreover, it was demonstrated that $A\beta_{1–42}$ administration did not cause a cognitive decline by using A_{2A} knockout mice [71], suggesting that $A\beta_{1–42}$-mediated toxicity in brain could be mediated, at least partially, through this receptor. In addition, A_{2A} blockade has not only been suggested to improve cognition, but also its ability to attenuate the neuroinflammation is very well known [73–75], whose effects clearly enhance the progression of disease. In this case, we might suggest that the desensitization of A_{2A} signalling observed after RSV treatment could also ameliorate the neuroinflammation in neurodegeneration. Taking into account that a lower $A\beta_{1–42}$ level after caffeine [69] and RSV [26] intake in mice was reported, we could suggest that RSV action might involve adenosinergic system. Moreover, RSV has potent antiatherosclerotic effects in vitro and in vivo where adenosinergic mechanisms may play a role, as pharmacologic blockade of the adenosine A_{2A} receptor with ZM-241385 eliminated beneficial effects of RSV on cholesterol efflux [76]. Interestingly, we have demonstrated that cholesterol itself is able to directly bind to A_{2A} receptors at the cell plasma membrane and possibly modulating A_{2A} function [77].

The increase in A_1 levels and their corresponding mediated signalling caused by RSV could enhance the neuroprotection of this compound by controlling the neurotransmitter release

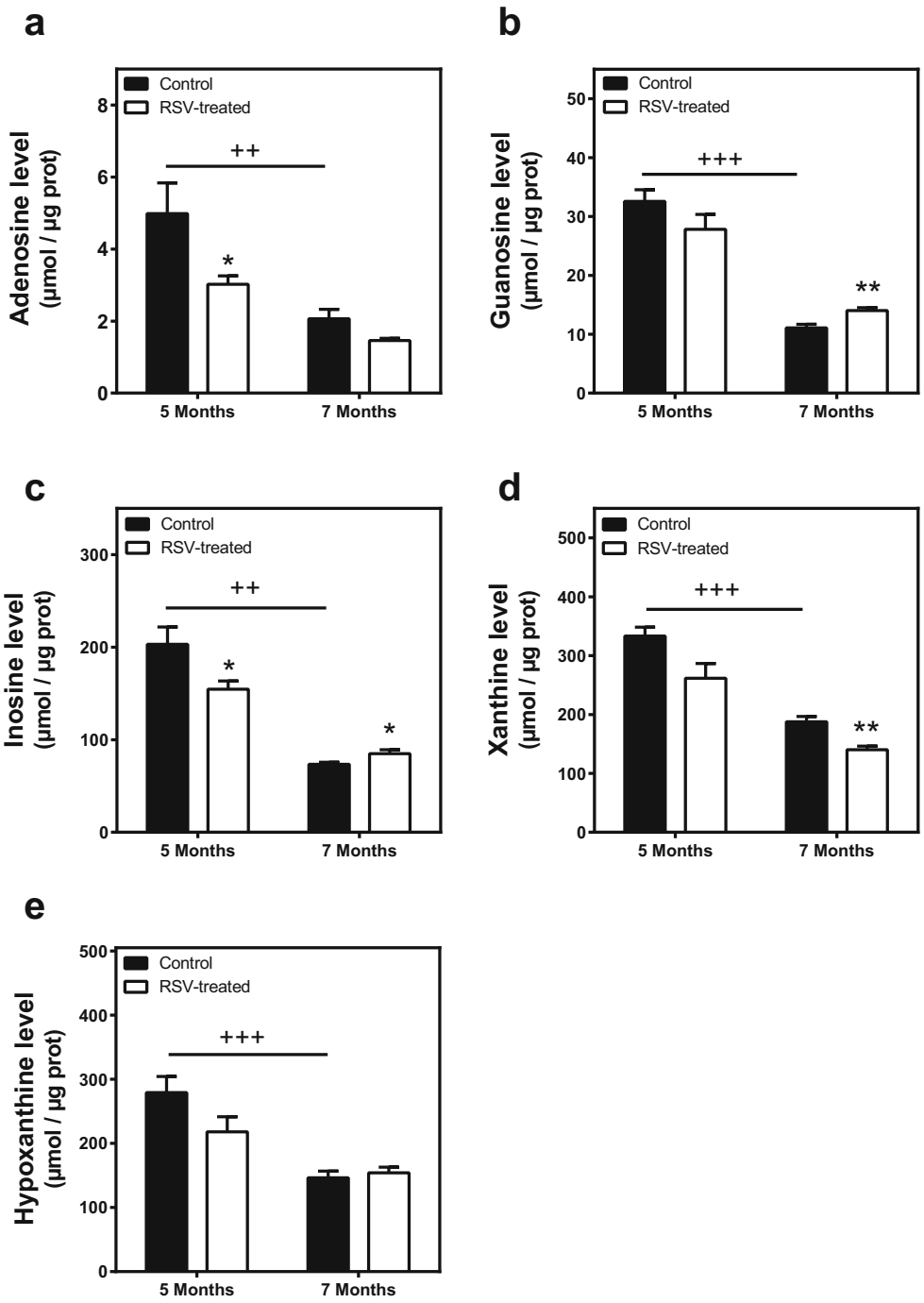


Fig. 8 Effect of RSV treatment on adenosine and its metabolite level. Adenosine (**a**), guanosine (**b**), inosine (**c**), xanthine (**d**), and hypoxanthine (**e**) levels were detected by using high-performance liquid chromatography (HPLC) as described in "Materials and Methods" section. Values are expressed as mean ± SEM of 59 different samples. $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$ significantly different from their corresponding control and $++p < 0.01$ and $+++p < 0.001$ significantly different from 5-month-old mice, all according to Student's *t* test

such as glutamate and dopamine. Glutamatergic [78–80] and dopaminergic [81, 82] systems are dysregulated in neurodegeneration. This upregulation of A_1 could promote an inhibition of glutamate release, and thus a reduction of glutamate-mediated excitotoxicity in neurodegeneration, as widely reported [83]. In agreement, some authors demonstrated that RSV treatment increased astrocyte glutamate intake [84], and reduced the excitotoxicity effects in epilepsy [85], where RSV has shown a protective effect [85–87]. Therefore, the resensitization of A_1-mediated signalling observed in our results might play a relevant role in this process. Based on the exploration of the possible therapeutic potential of A_1-mediated signalling, some studies aimed to target A_1 through specific agonists with no satisfactory results due to the rapid desensitization and downregulation of this receptor, as described in cortical cultured neurons [88] and rat brain [89], and the consequent side effect of this regulatory process.

It is widely known that adenosine controls the neurotransmission in both physiological and pathological conditions [6, 90]. In the present work, adenosine levels were found to be

decreased in age-related manner, which is in agreement with a lower 5′-nucleotidase enzymatic activity, suggesting that adenosine production could be significantly reduced during aging. Similar data were recently reported by our group in post mortem brain from AD patients in which it was found increased adenosine levels in the frontal cortex, and a lower 5′-nucleotidase enzymatic activity in this cortical area [2]. Nevertheless, RSV treatment seems to reduce the adenosine and inosine levels probably due to the lower activity of both 5′-nucleotidase and adenosine deaminase in treated mice when compared to their corresponding controls. In contrast, it has been reported an increase in 5′-nucleotidase and adenosine deaminase activities after RSV treatment in diabetic rats [91].

In summary, RSV treatment reversed the adenosine receptor modulation during aging. While an increase in A_1 receptors and their corresponding signalling pathway was detected, the contrary effect was observed in the case of A_{2A} and their corresponding functionality. Results described herein suggest a possible neuroprotective mechanism of RSV through adenosine signalling. Targeting adenosine-mediated signalling could be a therapeutic strategy for neurodegenerative diseases, and the role that this polyphenol plays via adenosinergic system should be considered.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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